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EFFECTS OF SINUS GLAND EXTRACTS ON LARVAL MOLTING AND ECDYSTEROID TITERS OF THE AMERICAN LOBSTER, *HOMARUS AMERICANUS*

MARK J. SNYDER AND ERNEST S. CHANG[1]

Bodega Marine Laboratory, University of California, P. O. Box 247, Bodega Bay, California 94923

ABSTRACT

Eyestalk ablation of 2nd stage larvae of the lobster, *Homarus americanus,* resulted in an accelerated rise in whole larval ecdysteroid titers during the 3rd stage. The appearance of both the premolt ecdysteroid peak and the subsequent ecdysis were accelerated by eyestalk removal during the previous molt interval. Replacement therapy, using sinus gland (SG) extracts from juvenile lobsters injected into 3rd stage larvae (ablated during the 2nd stage), delayed the next molt. This delay was significantly longer than for larvae injected with non-sinus gland (NSG) eyestalk tissue extracts. Sinus gland extracts also decreased ecdysteroid titers of ablated larvae within 12 h. Basal levels were maintained in SG-injected larvae while control larvae reached the premolt peak. These results indicate that a molt-inhibitory mechanism similar to that of juvenile and adult decapod crustaceans may also exist in lobster larvae.

INTRODUCTION

Although much information has been gathered on juvenile and adult crustacean endocrinology, relatively little is known about hormonal regulation during the larval stages. Several studies have been concerned with the histological examination of eyestalk structures during larval development. The X-organ is present in all larval stages of the lobster *Homarus americanus,* but the sinus gland is not apparent until the 3rd stage (Pyle, 1943). Both structures are present in all stages of the shrimp *Palaemon macrodactylus* (Little, 1969), but in five species of the shrimp *Palaemonetes* the sinus gland is not apparent until the 5th stage and is probably not functional until the postlarval stages (Hubschman, 1963).

The role of ecdysteroids in larval insects has been heavily investigated (Koolman and Spindler, 1983, for review). Their role(s) in larval crustaceans is less well understood. McConaugha (1979) reared 1st and 3rd stages of the crab *Cancer anthonyi* in seawater containing 20-hydroxyecdysone. Accelerated molting in the presence of the hormone was not observed and a large percentage of the surviving larvae had abnormal morphological characteristics. Molting is accelerated in 4th stage *H. americanus* by injection of ecdysone or 20-hydroxyecdysone during early proecdysis (Rao *et al.,* 1973). Another indication of ecdysteroids acting as molting hormones in crustacean larvae is the demonstration of the characteristic cyclical pattern of larval lobster ecdysteroid titers coinciding with molting events during the first three stages (Chang and Bruce, 1981). The predominant hormone at the premolt titer peak was 20-hydroxyecdysone. In addition, a putative Y-organ has been identified in a larval crab (McConaugha, 1980).

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[1] To whom all correspondence should be sent.

Abbreviations: sinus gland (SG), non-sinus gland eyestalk tissue (NSG), molt-inhibiting hormone (MIH).

Mechanisms that regulate larval crustacean molting cycles are poorly known relative to those of postlarval stages, although similar types of eyestalk ablation studies have been performed. Molting to the 1st crab stage is accelerated in megalopae of the blue crab, *Callinectes sapidus,* if both eyestalks are removed prior to a critical period (Costlow, 1963). Bilateral ablation during zoeal stages of the crabs *Sesarma reticulatum* (Costlow, 1966a) and *Pisidia longicornis* (Le Roux, 1979) has no effect on the duration of these stages. However, molting in the megalops stage is accelerated. American lobsters undergo precocious molting if ablated in the 4th (megalops) stage (Rao *et al.,* 1973). Zoeal molt cycle abbreviation was recently observed in lobsters by eyestalk extirpation (Charmantier *et al.,* 1985). In other studies, however, zoeal molting periods were unaffected by this treatment (Hubschman, 1963; Costlow, 1966b; Little, 1969). From earlier studies, Costlow (1968) inferred that the adult molt-controlling mechanism (molt-inhibiting hormone, MIH) was not operative in larvae. This idea was re-evaluated by Freeman and Costlow (1980). They proposed the existence of MIH in larvae of the crab *Rhithropanopeus harrisii,* based on observations of molt cycle abbreviation by eyestalk removal.

We report the results of experiments designed to investigate the presence of MIH-like activity in larvae of *Homarus americanus.* Previously, it was determined that eyestalk ablation of these larvae abbreviates subsequent molt intervals (Snyder and Chang, 1986). In the present study, ablated larvae were injected with sinus gland extracts possessing known MIH activity as assayed in juvenile lobsters (Bruce and Chang, 1984). Subsequent effects of these injections on molt intervals and whole larval ecdysteroid titers indicate that MIH may act to inhibit ecdysteroid production by the larval molting gland.

Materials and Methods

Animal rearing and ablations

Larvae from American lobsters, *Homarus americanus,* were used in these studies. The sources of egg-bearing females were as stated previously (Snyder and Chang, 1986). Larvae were collected from a single day's hatch of a single female and stocked into individual plastic tray compartments. They were fed freshly hatched *Artemia* nauplii and reared at 18–20°C.

Ecdysteroid titers

Larvae were reared in compartments, and were either left intact or bilaterally eyestalk-ablated 24 ± 4 h after molting to 2nd stage (Snyder and Chang, 1986). In the first experiment, batches of 15 ablated and 15 control larvae were collected every 24 h. Groups of larvae were processed for radioimmunoassay (RIA) as follows. Larvae were blotted dry, weighed to the nearest 0.1 mg, and homogenized (20 strokes, Dounce homogenizer) in 1.25 ml of distilled water. The homogenate was precipitated with 3.75 ml of 100% ethanol (Gold Shield Chemical Co.) and centrifuged (5000 rpm, 10 min, Sorvall HS-4 rotor). The pellet was re-extracted with 1.0 ml of ethanol and centrifuged again. The combined supernatants were extracted twice with 0.75 ml of hexane. Samples of the aqueous layers were dried under vacuum and analyzed by RIA using the I-2 (17-week bleeding) antiserum of Horn *et al.* (1976) using the procedures detailed by Chang and O'Connor (1979). Samples were taken from hatching through the approximate midpoint of the 4th stage.

The second experiment involved larvae that were destalked 24 ± 1.5 h after molt to 2nd stage. Three samples, each consisting of 2 or 3 larvae, were taken every 12 h

after eyestalks were removed until the end of the 3rd stage. Single homogenizations were performed in 0.25 ml of distilled water per 3 larvae. Methanol was added to a final concentration of 75%. Radioimmunoassays were performed after the centrifugation and hexane extraction steps. The antiserum used for these and all subsequent RIA determinations was the IB-4 serum, a gift from Dr. W. E. Bollenbacher, University of North Carolina. Statistical significance between ablated and control larvae was analyzed with Student *t*-tests. The extraction efficiency was checked by the addition of ^{3}H-ecdysone or ^{3}H-20-hydroxyecdysone (New England Nuclear) just prior to the homogenization steps. Mean efficiencies (for 4 determinations) were 85.3 ± 1.4% (ecdysone) and 86.7 ± 2.1% (20-hydroxyecdysone). Our data were not corrected for these extraction efficiencies.

Sinus gland injections

Sinus gland extracts from juvenile *H. americanus,* with molt intervals of 6–8 weeks, were prepared as described previously (Bruce and Chang, 1984). Molt staging of the juvenile donors was performed according to Aiken (1973). Only sinus glands from donors in the premolt stages were used. Eyestalks were removed and immediately chilled on ice. The sinus glands were dissected free from as much of the surrounding tissues as possible. Glands were pooled in a small drop of distilled water and homogenized in a micro-tissue grinder (Wheaton). The homogenate was lyophilized and stored at −75°C. Non-sinus gland extracts consisted of eyestalk tissue other than the sinus gland-X-organ complex. These tissues were estimated to be equivalent in size to the sinus glands utilized and were extracted in the same manner. Lyophilized extracts were reconstituted and used within 8 h.

Extracts were redissolved in sterile seawater, kept on ice for 10 min, and centrifuged for 2 minutes in a microcentrifuge (Beckman). The supernatant was reserved on ice for larval injections. Larvae were injected 24 ± 4 h (Exp. 1) or 24 ± 1.5 h (Exp. 2) after molt to the 3rd stage. In Exp. 1, both the intact controls and ablated larvae were injected with the extracts. Only ablated larvae were injected for Exp. 2. For the injections, the bevel of a #4 point, 33 gauge needle (Hamilton) was filed down to 25% of its initial length. Each injection contained 0.5 sinus gland (SG) or non-sinus gland (NSG) equivalent in 0.25 μl. Larvae were immobilized as for eyestalk ablation (Snyder and Chang, 1986), and injected once into the cephalothorax at a point just behind one of the fifth thoracopods. After allowance for initial blood clotting, larvae were returned to their rearing compartments. Lengths of the 3rd stage molt interval (and the 4th and 5th stage intervals for Exp. 1) were recorded every 8 h. For Exp. 2, the 3rd stage molt intervals were recorded every 2 h. Statistical comparisons were made with ANOVA and Scheffe analyses.

Injections and ecdysteroid titers

Larvae were destalked 24 ± 1.5 h after molt to 2nd stage as previously described (Snyder and Chang, 1986). The ablated larvae were randomly assigned to sinus gland-, non-sinus gland-, or non-injected groups. Injections were performed 24 ± 1.5 h after molt to 3rd stage as indicated previously. Triplicate groups of 2–3 larvae from each treatment were sampled by RIA every 6 h after injection of extracts until the molt to 4th stage. The mean values of each group at every time point were compared by Student *t*-tests.

Results

Ecdysteroid titers

The total ecdysteroid titers for batch extracts of whole larval lobsters is shown in Figure 1. Titers exhibited the characteristic cycling with each molt interval. There was an initial postmolt basal level followed by a premolt increase to an ecdysteroid peak, and a final decrease signaling the onset of ecdysis. Peak hormone concentrations during the 1st through 3rd stage molt intervals for intact and ablated larvae were similar. The similarity was apparent whether the data were expressed on a per larva or per 10 mg wet weight basis. However, during the 3rd stage, the time of appearance of the peak titer was significantly earlier for the ablated larvae than for the intact controls. An earlier premolt rise was also observed for the ablated larvae in the beginning of the 4th stage.

Further characterization of ablated and control larval ecdysteroid titers was accomplished by taking more frequent, multiple samples. These results are shown in Figures 2 (2nd stage molt interval) and 3 (3rd stage molt interval). Eyestalk ablation resulted in a small, non-significant, increase in ecdysteroid levels during the 2nd stage. No differences between hormone levels were obvious though ablated larvae molted to the 3rd stage significantly earlier. Titer differences were more apparent during the 3rd stage. There was a significantly earlier rise in the hormone titers of ablated animals during the first day after ecdysis. In addition, the premolt ecdysteroid peak occurred significantly sooner in ablated larvae. These ablated larvae also displayed subsequent accelerated decreases of hormone concentrations culminating in earlier ecdyses.

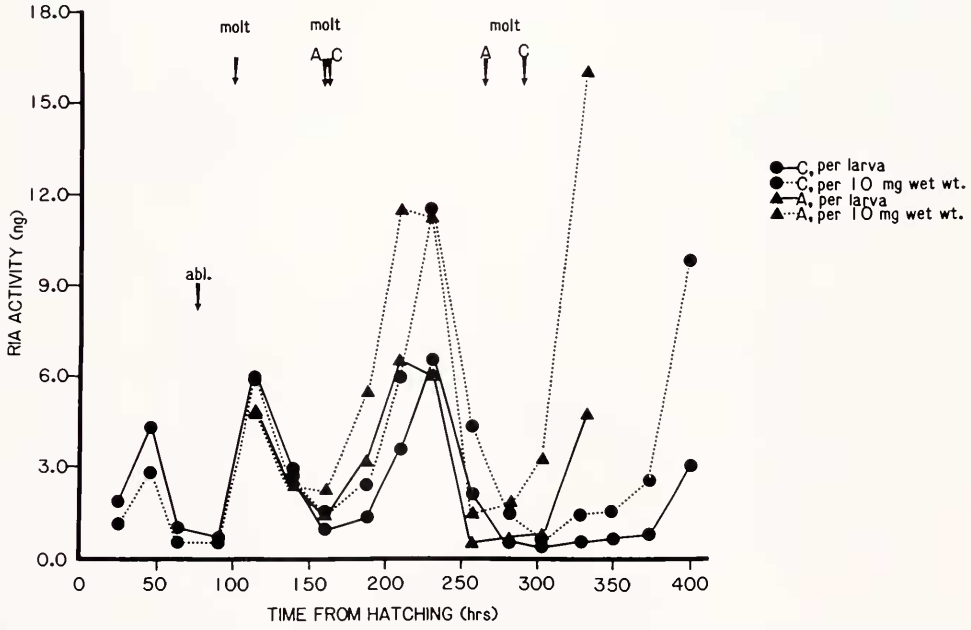

FIGURE 1. Ecdysteroid titers of eyestalk ablated (A) and control (C) lobster larvae from hatching through the midpoint of the 4th stage as determined by radioimmunoassay (RIA). Times of ablation (abl.) and molting are indicated by arrows.

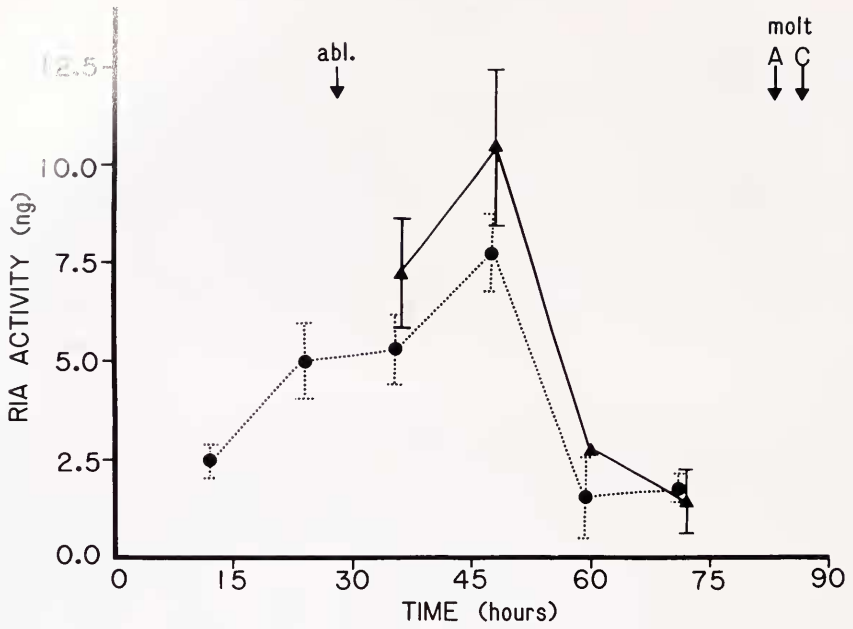

FIGURE 2. Ecdysteroid titers of eyestalk ablated (solid line) and intact control (dotted line) lobster larvae during the 2nd stage. Larvae were eyestalk-ablated (abl.) 24 ± 1.5 h after molt to 2nd stage. Symbols A and C refer to the time of molt to the 3rd stage for ablated and control larvae, respectively. RIA activity is reported on a per 10 mg wet weight basis for 3 samples of 2–3 larvae at each point. Bars are standard deviations of the 3 measurements.

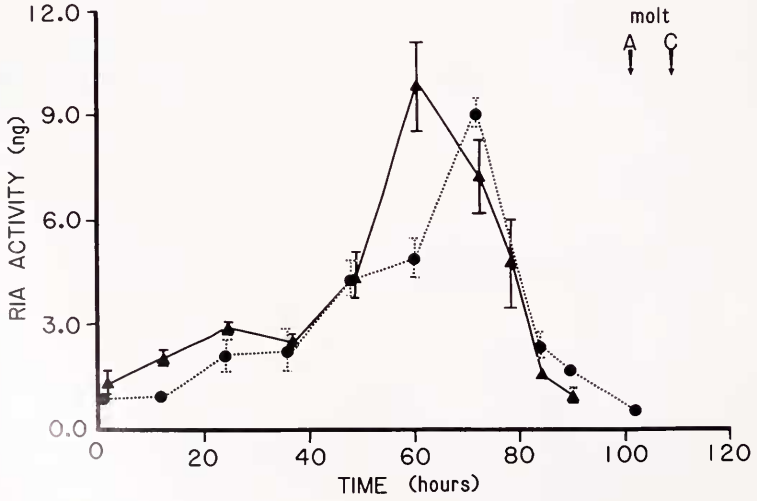

FIGURE 3. Ecdysteroid titers of eyestalk-ablated (solid line) and control (dotted line) lobster larvae during the 3rd stage. Larvae were eyestalk-ablated 24 ± 1.5 h after molt to 2nd stage. Symbols A and C refer to the times of molt to the 4th stage for ablated and control larvae, respectively. RIA activity is reported on a per 10 mg wet weight basis for 3 samples of 2–3 larvae at each point. Bars are standard deviations of the 3 measurements.

Injections and molting

Survival of extract-injected larvae was typically >75%. At 12–16 h after SG injection, blue coloration of the carapace was observed in ablated larvae, indicating that the injection had been successful. The color persisted for nearly 36 h, fading gradually. Non-SG injections had no such effect and the carapace remained white.

An indication of impending ecdysis could be made by the following observation. Within 12 h prior to a molt, larvae assumed an inverted position with the abdomen flexed dorsally towards the cephalothorax. This assisted in predicting subsequent molting events and hence, permitted closer observation.

Figure 4 shows the distribution of the 3rd stage larval molt intervals for the various treatments. It was observed that ablated larvae were significantly affected by the SG injections. There was a molt-delaying effect compared to both the ablated-non-injected ($P < 0.001$) and ablated-NSG-injected ($P < 0.05$) larvae. A portion of the molt interval delay in NSG-injected larvae could be related to the effects of the injection process alone. This is supported by observations that sterile seawater injections also delayed molt in some larvae (data not shown). Control, intact larvae were not significantly affected by SG injection. Further experiments concentrated on injection effects for ablated larvae only. The next two molt intervals of the injected larvae were followed. Table I shows that the injections (administered during the 3rd stage) had no effect on the subsequent 4th and 5th stage molt intervals.

To determine 3rd stage molt intervals more accurately, individual larvae were monitored every 2 h for molt to 4th stage. Molt intervals (in h) are given for this experiment in Table II. Sinus gland-injected larvae displayed molt interval lengths that were significantly longer than other groups ($P < 0.001$).

Injections and ecdysteroid titers

The above experiments demonstrated that injection of juvenile lobster sinus gland extracts increased the length of the molt interval. This phenomenon was investigated further by observing the ecdysteroid titers of SG-injected-, non-SG-injected-, ablated-non-injected-, and intact control larvae. Figure 5 shows the titers for all four groups.

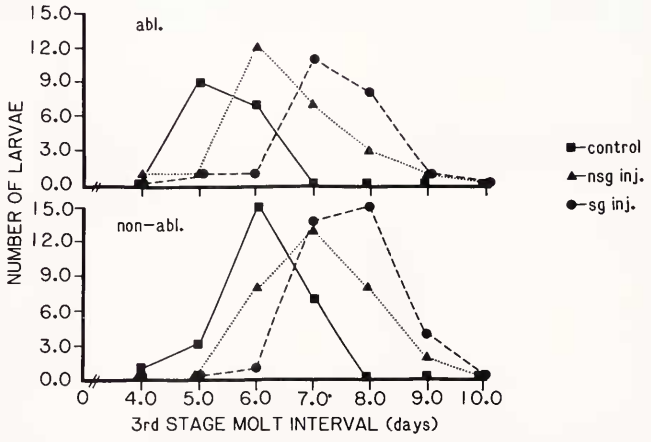

FIGURE 4. Distribution of lengths of 3rd stage molt intervals for ablated (abl.) and control (non-abl.) injected lobster larvae. Squares represent non-injected, triangles represent non-sinus gland (NSG) injected, and circles represent sinus gland (SG) injected larvae, respectively.

TABLE I

Length of 4th and 5th stage molt intervals of lobster larvae injected at 24 h after molt to 3rd stage

Treatment[a]	4th Molt interval (days)			5th Molt interval (days)		
	Mean	S.D.	n	Mean	S.D.	n
ES⁻	10.5[b]	1.1	15	10.8[b]	1.4	15
ES⁻, NSG	10.4[b]	1.2	21	11.1[b]	1.3	21
ES⁻, SG	10.3[b]	1.3	20	11.2[b]	1.0	20
C	13.7	2.0	19	12.8	0.9	19
C, NSG	14.0	1.4	35	12.5	1.1	33
C, SG	14.1	1.7	32	12.8	1.4	32

[a] ES⁻ and C denote eyestalk-ablated and intact control larvae, respectively. SG and NSG denote larvae that were injected with either extracts of 0.5 sinus glands or with an equivalent amount of non-sinus gland eyestalk material, respectively.

[b] Denotes significance from control groups at $P < 0.001$; within group differences were not significant for eyestalk-ablated and control larvae.

Within 12 h of injection, SG extracts caused a decline in whole larval ecdysteroid levels. A low basal titer similar to normal postmolt levels was maintained until after the other groups had begun a late premolt titer decline. The SG-injected peak titer was slightly lower than that of the ablated-non-injected larvae and was delayed about 12 h from the NSG-injected group. These effects of sinus gland injections were both significant and reproducible. Although NSG injection seemed to cause a titer increase above the other groups in Figure 5, this effect was not statistically significant nor was it repeatable in other experiments. The molt intervals of SG-injected larvae were significantly longer than in any of the other groups.

DISCUSSION

Molting hormone titers

It has been shown previously that eyestalk ablation of 2nd stage larval lobsters accelerates molt intervals of subsequent stages (Snyder and Chang, 1986). Ecdysteroid

TABLE II

Length of the 3rd stage molt interval of lobster larvae injected at 24 h after molt to 3rd stage

Treatment[a]	3rd Molt interval (h)		
	Mean	S.D.	n
C	139.0	5.4	27
ES⁻	126.3[b,d,e]	4.6	15
ES⁻, NSG	137.3[c,e]	7.2	23
ES⁻, SG	151.7[b,c,d]	6.8	15

[a] Abbreviations for the various treatments are the same as for Table I.

[b] Denotes significance from non-ablated controls ($P < 0.001$).

[c] Denotes significance from ablated-non-injected group ($P < 0.001$).

[d] Denotes significance from ablated-NSG-injected group ($P < 0.001$).

[e] Denotes significance from ablated-SG-injected group ($P < 0.001$).

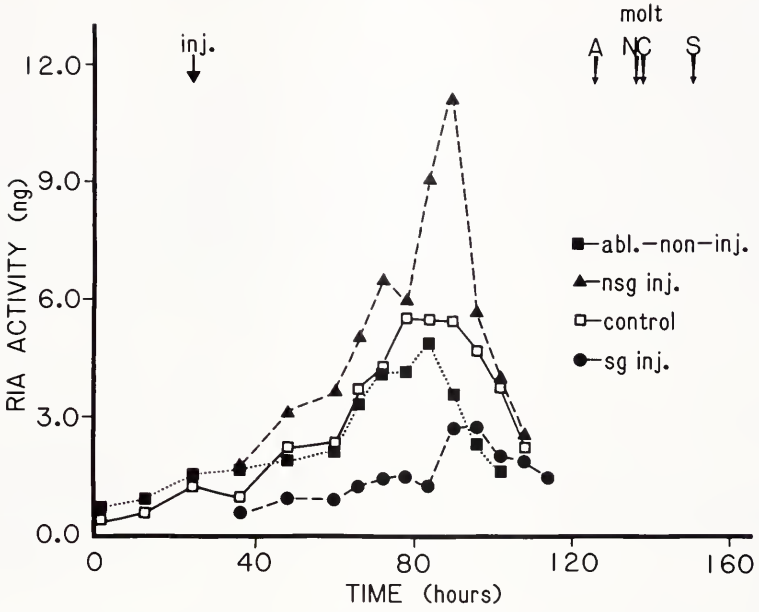

FIGURE 5. Ecdysteroid titers (S.D. bars removed for clarity) of ablated-injected, non-injected, and intact control lobster larvae during the 3rd stage. Eyestalk-ablated larvae were injected (inj.) with 0.5 sinus gland (SG) or non-sinus gland (NSG) equivalents at 24 ± 1.5 h after molt to 3rd stage. Symbols A, N, C, and S refer to the times of molt to 4th stage for ablated-non-injected (solid squares), ablated-NSG-injected (triangles), intact controls (open squares), and ablated-SG-injected larvae (circles), respectively. Larvae were ablated 24 ± 1.5 h after molt to 2nd stage.

titering of ablated 2nd stage larvae indicates a slight, insignificant increase during the premolt peak titer (Fig. 2). However, a statistically significant effect is observed in lobster larvae during the 3rd stage. A greater postmolt increase and an earlier premolt peak occur in ablated larvae during the 3rd stage. Concomitant with this effect is the observation of significantly abbreviated 3rd stage molt intervals. This is the first demonstration of the effect of ablation on the molting hormone titer in crustacean larvae. It is a very similar effect to that seen in juvenile lobsters that immediately display a premolt titer increase upon eyestalk ablation (Chang and Bruce, 1980).

These results support the idea of the presence of molt-inhibiting hormone (MIH) activity in decapod larvae, at least after the 3rd stage in *H. americanus*. First stage larvae do not survive ablation so it is difficult to ascertain the point of onset of MIH activity. The evidence suggests that the lack of an MIH-like factor(s) affects only the relative position of the premolt peak during the molt interval. These observations of the hormone titers of 1st and 2nd stage larvae and those by Chang and Bruce (1981) suggest that it may not be possible to further shorten the onset of the premolt ecdysteroid peak. The titer begins to rise in the typical premolt manner within 12 h of the previous molt in both stages.

Since ecdysteroid titers continue to cycle in eyestalk-ablated larvae, the question of the ultimate control of molting gland activity becomes apparent. In the absence of MIH, there must be an alternative mechanism for the control of ecdysteroid production. This concept has been reviewed by Skinner (1985). In addition, ablated juvenile lobsters that are well-fed continue to display cycling hormone titers and accelerated molting until they die (Chang, 1985). Results reported here with larval lobsters, as well as data

on juvenile and adult crustaceans, suggest that ecdysteroid production may be controlled by an additional factor.

Injection effects on molting and edysteroid titers

Data reported here show that bilateral ablation of eyestalks accelerates the molt cycle in *H. americanus* larvae. Injection of juvenile lobster sinus gland (SG) extracts into these larvae at 3rd stage postmolt significantly delays the following molt (Fig. 5, Table II). Sinus gland tissue extracts delay molting significantly greater than non-sinus gland (NSG) extracts. This is the first demonstration of an effect of previously determined MIH-active substances (Bruce and Chang, 1984) on crustacean larvae. The slight molt-inhibition observed in NSG-injected compared to ablated-non-injected larvae may be due to an injury effect. Alternatively, the NSG tissues may have included small sections of the X-organ nerve tracts that contain MIH. In this instance, a very slight amount of MIH may have contributed to the molt delay.

Injections of extracts into intact larvae also were effective in delaying molt. There was not a specific SG effect however. This may have been due to the rapid degradation of the injected MIH factor(s) such that the molt delay could be attributed solely to an injury effect. An explanation for the lack of an enhanced inhibitory effect of SG relative to NSG injections is that endogenous hormone occupies most of the available MIH receptor sites. The injected hormone may then be degraded before the hormone receptors become available.

The results of extract injections on ecdysteroid titers support the data concerning the molt delay effect. Sinus gland injections depressed the ablated larval titers to basal levels within 12 h (Fig. 5). This effect was reproducible and more significant than observed in juveniles (Bruce and Chang, 1984). It is possible, therefore, that 3rd and 4th stage lobster larvae have a molt-inhibitory control mechanism(s) similar to that of juvenile and adult crustaceans. Pyle (1943) studied the X-organ-sinus gland system in larval lobsters using histological techniques. The X-organ was present in all larval stages, but the sinus gland was not found until the 3rd stage. Though Pyle found no evidence of neurosecretion in his study, the present results support the possibility that MIH is secreted from the eyestalk X-organ-SG complex during larval lobster development.

Sinus gland extract injections reduced ecdysteroid titers in the crab *Pachygrapsus crassipes* (Keller and O'Connor, 1982), and the ecdysone secretion of subsequently cultured Y-organs from ablated donor crayfish, *Orconectes limosus* (Gersch *et al.*, 1980). Sinus gland extracts administered *in vitro* reduced biosynthetic activity of cultured crayfish (Gersch *et al.*, 1977) and crab (Soumoff and O'Connor, 1982) Y-organs. All of these reports, in addition to our results on lobster larvae, suggest that the MIH factor(s) from the X-organ-SG acts upon the activity of ecdysteroid-synthesizing tissues to inhibit molting. The MIH factor(s) may also, however, act directly upon ecdysteroid target tissues (Freeman and Bartell, 1976; Freeman and Costlow, 1979).

In summary, it appears that regulation of larval decapod molting occurs in a similar manner to that found in juveniles and adults. Eyestalk removal results in both an earlier premolt ecdysteroid peak and the subsequent molt. Injection of juvenile lobster sinus gland extracts into destalked larvae delays the following molt. The SG injections also quickly diminish the whole larval ecdysteroid titer. The control of larval molting by an MIH-like mechanism which regulates ecdysteroid production is thus indicated. Future work will clarify the role(s) played by eyestalk neurosecretory centers in the regulation of larval molting processes. The rapid molt cycles of lobster larvae (about five days at 20°C for 3rd stage larvae) indicate that these animals are a good whole animal bioassay for the study of sinus gland peptides, in particular, MIH.

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